ELASTIC AREA COMPRESSIBILITY MODULUS OF RED CELL MEMBRANE

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ABSTRACT Micropipette measurements of isotropic tension vs. area expansion in preswollen single human red cells gave a value of 288 ± 50 SD dyn/cm for the elastic, area compressibility modulus of the total membrane at 25°C. This elastic constant, characterizing the resistance to area expansion or compression, is about 4×10^4 times greater than the elastic modulus for shear rigidity; therefore, in situations where deformation of the membrane does not require large isotropic tensions (e.g., in passage through normal capillaries), the membrane can be treated by a simple constitutive relation for a two-dimensionally, incompressible material (i.e. fixed area). The tension was found to be linear and reversible for the range of area changes observed (within the experimental system resolution of 10%). The maximum fractional area expansion required to produce lysis was uniformly distributed between 2 and 4% with 3% average and 0.7% SD. By heating the cells to 50°C, it appears that the structural matrix (responsible for the shear rigidity and most of the strength in isotropic tension) is disrupted and primarily the lipid bilayer resists lysis. Therefore, the relative contributions of the structural matrix and lipid bilayer to the elastic, area compressibility could be estimated. The maximum isotropic tension at 25°C is 10–12 dyn/cm and at 50°C is between 3 and 4 dyn/cm. From this data, the respective compressibilities are estimated at 193 dyn/cm and 95 dyn/cm for structural network and bilayer. The latter value correlates well with data on in vitro, monolayer surface pressure versus area curves at oil-water interfaces.

INTRODUCTION

Expansion of the mammalian red blood cell membrane during osmotic swelling of the cell has been investigated and discussed for at least four decades (Ponder, 1971). Ponder's initial estimates indicated little change in surface area. Katchalsky et al. (1960) deduced radius changes of 8% and surface area increases of the order 20% from cell volumes calculated from osmotic swelling data (see Ponder, 1971). Subsequent single cell observations by Rand and Burton (1963) showed similar increases of 10–20%. However, the optical technique used by Rand and Burton were subject to significant uncertainties due to the defocusing method employed in their measurements. Quantitative optical processing was developed to provide adequate accuracy in measuring surface area and volumes of red cells by Evans and Fung (1972). For a cell population, Evans and Fung (1972) found a 7% increase in surface area of sphered cells and no measurable changes in surface areas of cells at intermediate states of swelling. In order to avoid statistics of cell populations, Evans and Leblond (1973)

measured surface areas and volumes of osmotically swollen single cells and found no detectable surface area changes (accuracy limited to 2%); however, it was observed that these individual cells were not completely spherical and attempts to obtain spherical cells only resulted in lysis.

It was apparent from these studies that the red cell membrane is highly resistant to changes in surface area. On the other hand, large membrane extensions at constant area are easily produced (Rand and Burton, 1963; Hochmuth and Mohandas, 1972; Evans and LaCelle, 1975). This compound evidence led to a new concept for red cell membrane as a highly deformable, two-dimensional material with very small area compressibility (Skalak et al., 1973; Evans, 1973*a*). Correlation of the hyperelastic, extensional behavior observed in micropipette and fluid-shear deformation of red blood cells was achieved using the material constitutive relation which presumes that the surface is infinitely resistant to area change, two-dimensionally incompressible (Evans, 1973*b*; Evans and LaCelle, 1975). The result of the analyses of the two types of experiments was an elastic modulus that characterizes extension at constant area (i.e. related to shear rigidity); the value of the shear modulus was measured to be 7×10^{-3} dyn/cm.[1] The validity of the area incompressibility assumption is determined by the ratio of the elastic modulus, which characterizes area compression and expansion, to the shear modulus; if this ratio is large and if the membrane is not subjected to large, in-plane isotropic tensions, then the reduced form of the elastic constitutive relation is appropriate. In this case, the principal tensions are given by,

$$T_{11} = -p_m + (\mu/2)(\lambda_1^2 - 1),$$
$$T_{22} = -p_m + (\mu/2)(\lambda_2^2 - 1), \tag{1}$$

where $-p_m$ is the locally isotropic membrane tension (analogous to hydrostatic pressure in bulk materials), μ is the elastic modulus characterizing shear resistance of rigidity, and (λ_1, λ_2) are the principal material extension ratios. Fig. 1 illustrates the behavior of an intrinsic membrane material element.

For the situation of osmotic swelling and lysis,[2] large isotropic tensions are developed which preclude the use of Eqs. 1 and which require the inclusion of an area compressibility term. Since it will be shown that the area modulus is significantly larger than the elastic shear modulus, the isotropic tension component in Eq. 1 can be simply expressed by,

$$-p_m = K \cdot \Delta\alpha, \tag{2}$$

[1] The elastic constants of a two-dimensional material have the appropriate units of force per unit length and not stress units of force per unit area (see Evans, 1975*a* for discussion).

[2] Lysis is membrane failure in isotropic tension (Evans, 1975*b*). It is failure in the sense that the interior contents "escape," but not necessarily complete material destruction. It is well known that osmotically lysed cells reseal to form ghosts. Lysis is unlike the fragmentation of cells (i.e. plastic failure in shear) which occurs at much lower membrane force levels and results in loss of material (Evans and Hochmuth, 1976*b*).

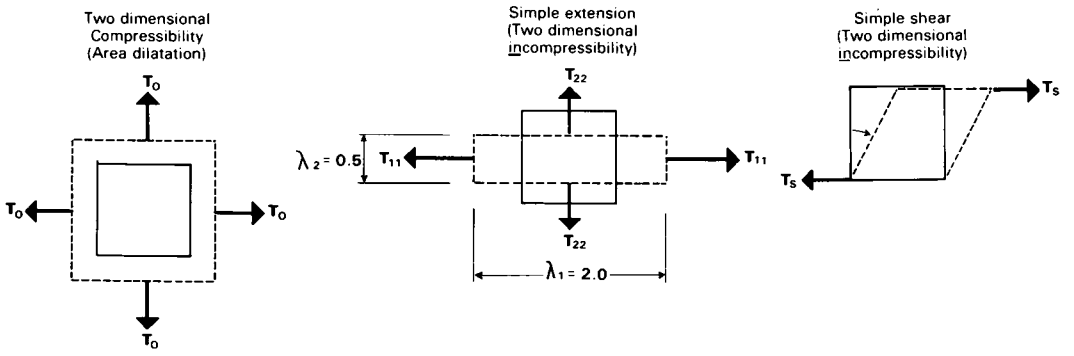

FIGURE 1 Schematic illustration of the intrinsic deformations of a membrane element in response to applied tensions. *Left:* Area expansion for isotropic tension. *Middle:* Simple extension at constant area. *Right:* Simple shear at constant area. Both extension and shear are the same deformation when the coordinate systems are properly chosen (and if the material is isotropic in the plane of the membrane).

where K is the area compressibility modulus and $\Delta\alpha$ is the fractional change in area $\Delta A/A_o$ as measured from the natural membrane state (Evans, 1975*a*). Eq. 2 is a first order, linear representation which is used for small strains ($\sim 1\%$) and must be verified by experiment. When large isotropic tensions exist, the elastic shear contribution can be neglected and the principal tensions are essentially equal,

$$T_{11} \simeq K \cdot \Delta\alpha,$$
$$T_{22} \simeq K \cdot \Delta\alpha. \tag{3}$$

In this paper, we present experiment data for micropipette aspiration of osmotically, preswollen red cells that provide the elastic relationship between isotropic tension and area expansion in human red cell membranes. From this relationship, the elastic area compressibility modulus, K, is calculated; the maximum area dilation for immediate cell lysis is determined along with the maximum tension required to produce immediate lysis at 25°C. When cells are heated to above 45°–50°C, large vesicles are produced (Bessis, 1973; Ponder, 1971) indicating that in parts of the membrane, the lipid bilayer remains as the primary structure of the membrane. Lysis tensions at this temperature provide an estimate of the lipid bilayer area compressibility modulus. The free energy change required to "expose" the hydrophobic interior of the lipid bilayer during expansion can be calculated and compared with the "free energy density of transfer" of a hydrocarbon molecule to aqueous medium (Reynolds et al., 1974; Tanford, 1973).

EXPERIMENTAL METHODS

Venous blood from healthy donors was collected fresh each morning in a syringe containing a small drop of heparin. After removal of the plasma layer and "buffy" coat, the cells were washed twice in 300 mosM, phosphate-buffered saline (pH 7.4) and resuspended in a phosphate-buffered solution at 135 mosM to preswell the cells just

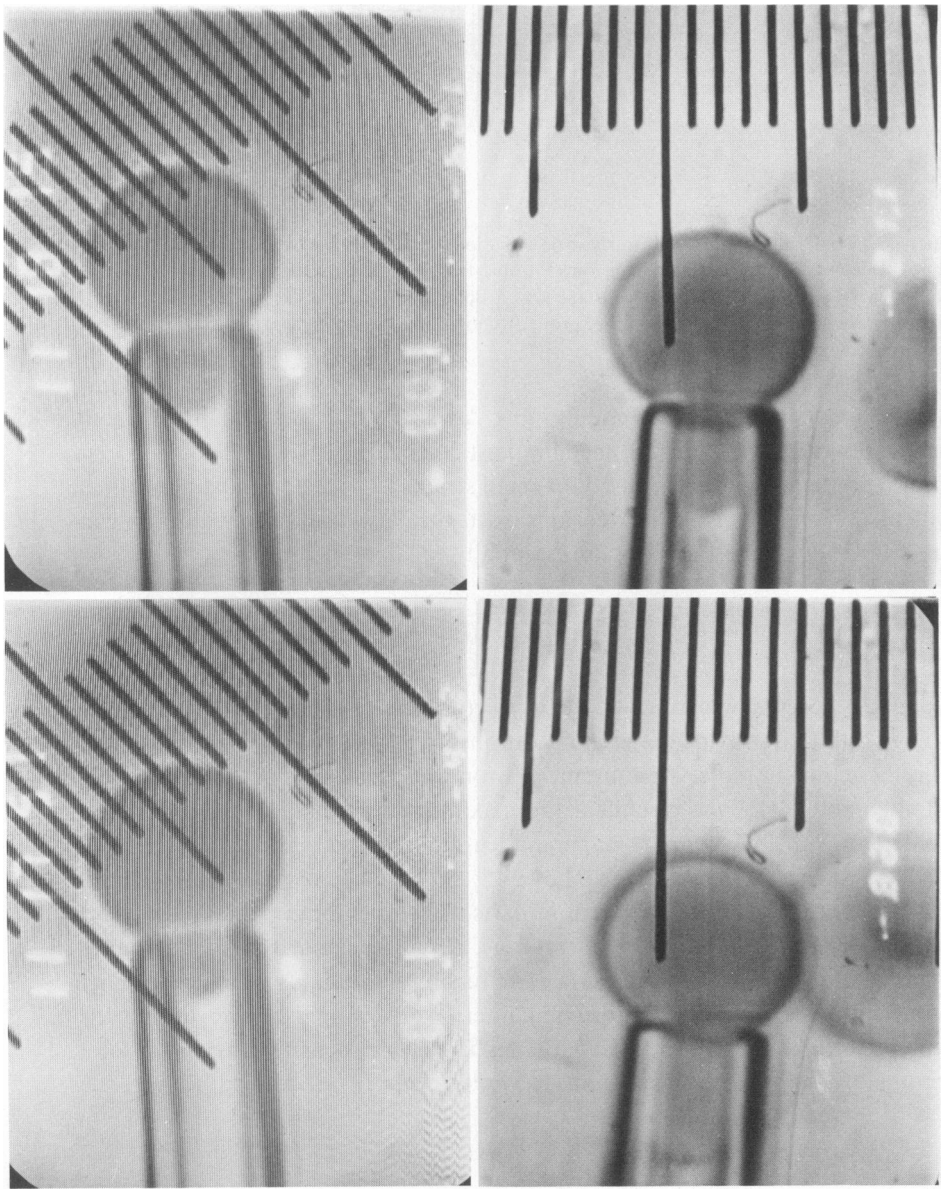

FIGURE 2 The increase in cell projection length for an increase in applied pressure is shown for the cell in the upper two photographs ($\Delta P \cong 4 \rightarrow 60$ mm Hg). The decrease in cell projection length for a decrease in applied pressure is shown for another cell in the lower two photographs ($\Delta P \cong 88 \rightarrow 21$ mm Hg). These photographs were taken directly from the television recording; the micrometer scale is in the eyepiece before the TV camera (10 div = 9 μm). The temperature was 25°C.

before the experiments were performed. All solutions contained 0.5% serum albumin (by weight) and were filtered. The cells (in very small concentrations) were injected into a small microchamber (1 cm × 2 cm × 0.1 cm) and were changed every 20–30 min.

The micropipette, aspiration system consisted of a pneumatically controlled micromanipulator (De Fonbrune type C) mounted on the stage of a Leitz inverted microscope (E. Leitz, Inc., Rockleigh, N.J.). The 1 mm glass pipette was pulled to a needle point and then broken by quick fracture to obtain a flat tip in the desired range of 1.8–2.4 μm. The pipette was coupled by a continuous water system to a micrometer positioned water manometer for zero pressure adjustment. The negative pressure was applied using a microsyringe coupled by air to the water manometer and the pressure was measured from the continuous water system with a digital pressure transducer (Celesco p90-D with 10^{-2} mm Hg resolution; Celesco Industries, Inc., Costa Mesa, Calif.). A temperature-controlled chamber was mounted on the insulated stage and was controlled by a small thermocouple placed in the microchamber itself. The temperature could be controlled to 0.1°C and the range was between 2°C and 60°C. A quartz-insulated, long working length 40× (0.6 NA) objective was used to avoid conduction from the chamber. Temperature, negative pressure, and time were simultaneously recorded on video tape using an amplitude mixed television system. Fig. 2 is a series of photographs of single "video" frames. The micrometer eyepiece (10 div = 9 μm) was always in the video, microscope image in order to record the changes in TV monitor horizontal and vertical sweep widths so that the system could be calibrated at the time of recording.

The osmotically, preswollen cells were deliberately kept slightly flaccid in order that the aspirated length of the cell projection was between one and two pipette diameters. This provided a method of leak detection since a water leak resulted in "necking" and pinching off of a small globule (fragmentation). If no leak occurs in the annulus surrounding the cell projection, then the circumferential or "hoop" tension divided by the pipette radius is always less than the transmembrane pressure difference in the cylindrical region and the membrane is forced against the pipette inner wall; lysis could always be easily differentiated from fragmentation because the cell disappeared faster than the TV framing speed ($\sim\frac{1}{30}$ s). In addition, small microspheres (of polystyrene or methylmethacrylate) were used to detect leaks but did not provide any improvement over observation of microfragmentation and movement of other cells and small elements in the vicinity of the pipette tip. Precaution was taken to insure that adhesion to the pipette glass did not occur along the projection (cleanliness is the most important factor; all solutions were filtered regularly and a 0.5% serum albumin level was maintained).

RESULTS AND ANALYSIS

Fig. 2 shows the increase in the aspirated length of a swollen cell as the pressure was increased. It was observed that the length increased linearly with pressure and that the reduction of pressure resulted in the reverse behavior with complete recovery. The cycle was repeatable until the cell lysed (most cells lysed during the first pressure in-

crease phase, depending on the magnitude of the membrane tension). The aspirated length responded to the applied pressure as fast as the overall system response time (the order of a 10th of a second). The rate of deformation of the membrane was, therefore, on the order of 0.1 s^{-1} (the isotropic rate of dilation or condensation of surface area). When the pressure was held at a specific value, no length change could be detected for periods on the order of a minute (for longer periods the high pressure produced water filtration and observable cell volume reduction). This implies that the membrane was responding elastically. Pressures producing tensions of 6 dyn/cm or greater usually resulted in lysis within 10 s and pressures producing tensions of 10–12 dyn/cm or greater resulted in instantaneous lysis.[3] Again, in no case was any change in length detected when the system was at constant pressure (except for volume losses when sublytic tensions were maintained for exceptionally long periods). Rand (1964) reported that "the cell in the pipette does not immediately appear to change when the high pressures are applied, and then hemolyzes and disappears suddenly into the pipette...." This observation apparently concerned Rand because he recognized that it was not consistent with the viscoelastic model that he chose to represent the temporal character of lysis. There were no obvious viscous effects (certainly, they exist but apparently the material dissipation and "creep" are not of first order importance in lysis where area expansion rates are a few percent per second).

The optical system diffraction limits the area resolution of the whole cell to the order of 10% (Evans and Fung, 1972). However, in the pipette, it is possible to detect small changes in area (assuming that the volume remains constant which is reasonable for the time periods involved). This is because the movement of the aspirated length *can* be measured accurately even though the absolute position of the "cap" of the cell projection cannot be specified. Using the contrast enhancement of the television system, cell projection movement on the order of 0.07 μm is observable; for a 2 μm diameter pipette, this represents an area change of 0.4 μm^2. The total surface area of the cell is on the order of 140 μm^2; therefore, the minimum detectable area change is about 0.3%. Since all measurements are made on individual cells, the results for fractional changes in area and the calculated elastic compressibility modulus are limited in accuracy by the measurement of total cell area and pipette diameter which implies about 10% of the measured values. The maximum area change, where immediate lysis occurred, was about 4% ($\pm$0.4%). Lysis occurred within about 20–30 s for area changes of about 2%.

Since the applied aspiration pressures produced membrane tensions greater than 1 dyn/cm, the shear rigidity terms in Eqs. 1 can be neglected (μ is of the order 10^{-2} dyn/cm and the extension ratios were less than 3:1). Therefore, the state of membrane

[3]The time of lysis appears to characterize a stochastic process with a mean "life time" that increases from zero for tensions of 10–12 dyn/cm to "infinity" for tensions of the order of 3–4 dyn/cm. The reversibility (i.e. the ability of the cell membrane to reseal and form a "ghost") and the broadened time distribution for smaller membrane area expansions indicate that the process is related to the lipid bilayer "surface" pressure and thermodynamic state.

tension is isotropic (the spherical shape of the portion of the cell outside the pipette is verification of the isotropy and uniformity of membrane tension). If no adhesion or friction exists between the pipette wall and cell membrane, then the membrane tension is the uniform over the total cell surface. For isotropic tension, the law of Laplace can be applied as the equation of equilibrium of the unconstrained membrane surfaces (i.e. the cap of the projection and the spherical portion outside the pipette).

$$P_i - P_p = 4T_o/D_p$$
$$P_i - P_o = 4T_o/D_c, \tag{4}$$

where P_i, P_p, and P_o are the fluid pressures inside the cell, in the pipette, and outside the cell, respectively. D_p and D_c are the pipette and outside, spherical segment diameters. T_o is the membrane isotropic tension.

$$T_o = K \cdot \Delta\alpha. \tag{5}$$

The cell pressure can be eliminated from Eqs. 4 to give a relation between the pipette aspiration pressure ($\Delta P \equiv P_o - P_p$) and the membrane tension (this relationship was also used by Rand, 1964).

$$T_o = (\Delta P \cdot D_p)/4(1 - D_p/D_c). \tag{6}$$

The increase in surface area is calculated from the increase in projection length and the assumption of constant cell volume.

$$\Delta A = \pi \cdot D_p \cdot \Delta L + \pi(D_c^2 - D_{c_o}^2), \tag{7}$$

where ΔL is the increase in cell projection length; D_{c_o} is the initial diameter of the spherical portion outside the pipette. Constant volume yields,

$$(D_c/D_{c_o})^3 = 1 - [(6D_p^2 \cdot \Delta L)/4D_{c_o}^3],$$

and the initial cell surface area is calculated using the following equation summing the projection surface area and the surface area of the outside spherical portion,

$$A_o = \pi \cdot D_p \cdot L + \pi[D_{c_o}^2 - (D_p^2/4)], \tag{8}$$

where L is the cell projection length. Using Eqs. 6, 7, and 8, the membrane isotropic tension vs. fractional increase in area $\Delta\alpha$ is determined from single cell data for Δp vs. ΔL. Figs. 3*a*, *b*, *c*, and *d* are typical cell tension vs. area expansion curves for maximum tensions of 6–11 dyn/cm at 25°C. In each case, the cell lysed when the maximum tension was reached. Fig. 4 is a histogram of the elastic, area compressibility moduli determined at 25°C from the slope of the tension-area expansion curve (established by a minimum of square errors). The mean value is 288 (± 50 SD) dyn/cm. This is about 4×10^4 times the elastic coefficient for shear rigidity; therefore, the membrane behaves in a two-dimensionally, incompressible manner in shear and elongation (as represented by Eqs. 1). The maximum fractional area expansion at lysis was *uniformly* distributed between 2 and 4% with an average of 3% and 0.7% SD.

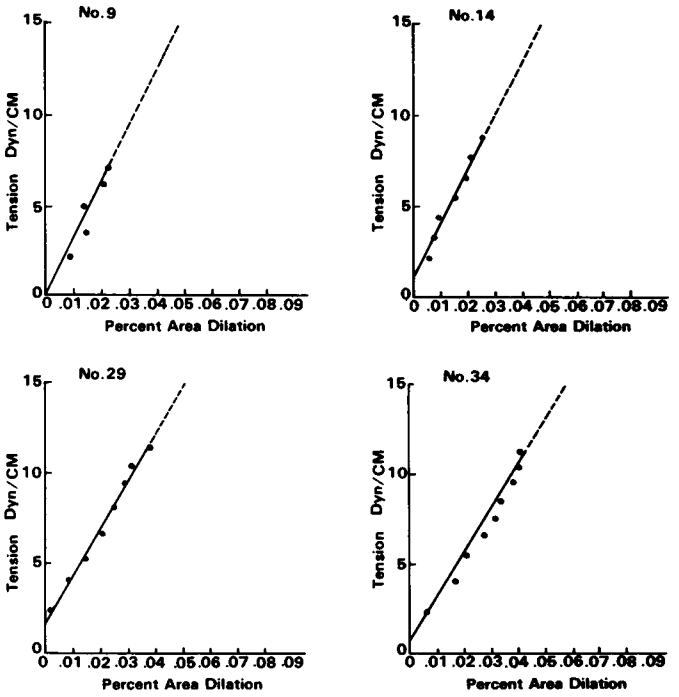

FIGURE 3 Red blood cell area compressibility. Plots of tension vs. area expansion are shown for four specific cells in the tension range of 6–11 dyn/cm. Each data point has an implicit 10% uncertainty. These cells lysed at the maximum tension data point. Data taken at 25°C.

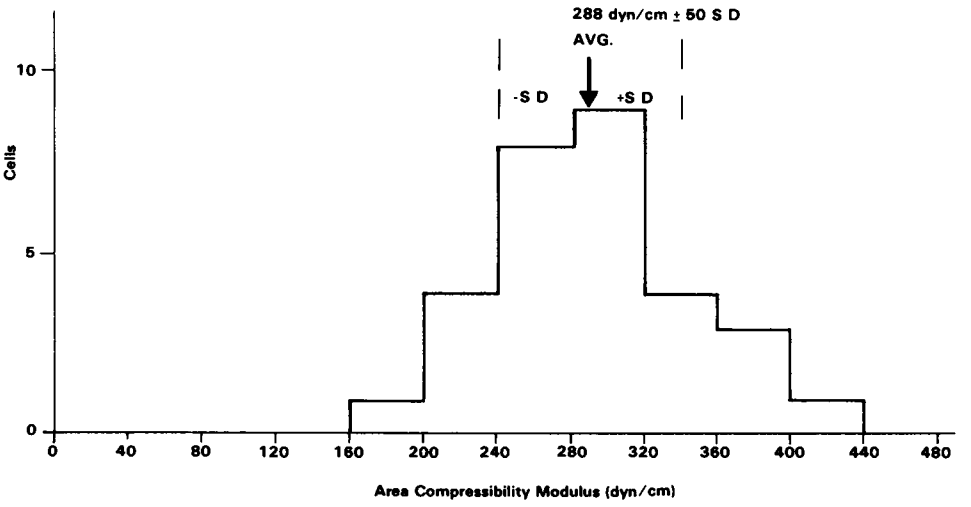

FIGURE 4 Histogram of the elastic, area compressibility modulus for a population of 30 cells at 25°C. The mean is 288 dyn/cm and standard deviation 50 dyn/cm.

DISCUSSION

Because the red cell membrane is a material composite of a fluid, lipid bilayer and structural matrix (perhaps spectrin) as discussed by Marchesi et al. (1970), Steck (1974), and others (mechanically verified by the elastic and viscous shear response by Evans and LaCelle, 1975; Evans and Hochmuth, 1976*a*,*b*), separation of the relative contributions by each element to the area compressibility can be estimated. This was accomplished by heating the cells to above 50°C where vesiculation takes place ("budding," Ponder, 1971). Vesiculation represents a loss of shear rigidity and a transformation of the surface into a two-dimensional liquid (stable only in a spherical configuration when unsupported). Clearly, it is not known whether all of the structural matrix was disrupted but the lysis tension measurements indicate the local areas of the membrane were weakened to the level of the lipid bilayer strength; failure will occur at the weakest locations. In addition, small vesicles (probably surrounded by lipid bilayer membranes) were observed to be attached to the cell surface; when the pressure was applied, the cell projection increased by incremental jumps as each vesicle recombined with the cell membrane. Finally, after all vesicles were reincorporated, immediate cell lysis occurred for tensions of 3–4 dyn/cm.

Assuming that the lipid bilayer and the structural matrix component of the membrane are tightly associated (i.e. experience the same area expansion and compression), the tension can be expressed as the sum of the two membrane contributions,

$$T_o = (K_N + K_{BL}) \cdot \Delta\alpha \tag{9}$$

where K_N and K_{BL} are the area compressibility moduli of the structural matrix and lipid bilayer, respectively.

Since lysis is determined by a change in membrane permeability, the maximum area expansion of about 3% probably characterizes the bilayer dilation for both red cells at 25°C and vesiculated cells at 50°C. If this is true, then the lipid bilayer area compressibility modulus would be about 33% of the total membrane area modulus and the structural matrix would provide the remaining 67% at 25°C (as estimated using the maximum tensions for immediate lysis at 25°C and 50°C).

$$K_{BL} \simeq 95 \text{ dyn/cm};$$

$$K_N \simeq 193 \text{ dyn/cm}.$$

The respective compressibility moduli are estimated at 193 dyn/cm and 95 dyn/cm for structural network and bilayer. The latter value correlates well with data on in vitro, monolayer surface pressure vs. area curves at oil-water interfaces (Tanford, 1973). From the monolayer data, the area compressibility modulus in the range of 50–60 $Å^2$ per molecule is about 50 dyn/cm; twice this value would be the bilayer area compressibility modulus, i.e. 100 dyn/cm.

The equilibrium of a lipid bilayer is determined by the balance of the constrictive effects of hydrophobic interactions against the area compression resistance or "surface pressure" (Evans, 1975*b*; Evans and Simon, 1975). The work required to expand the lipid bilayer is given by (per unit area),

$$\Delta W_{BL} = (K_{BL} \cdot \Delta\alpha^2)/2. \quad (10)$$

Therefore, it is possible to estimate the free energy per mole of membrane lipids that was required to produce instant lysis. (We will use 0.4×10^{-9} mol/cm^2 as the area concentration of lipids, see Cooper, 1970).

$$\Delta W_{BL} = 95(2.4 \times 10^{-8})(0.03)^2/(0.8 \times 10^{-9})$$

$$\Delta W_{BL} \sim 3 \text{ cal/mol}.$$

The free energy required to create 1 Å^2 of pure water-hydrocarbon (hydrophobic) interaction is given by Tanford (1973) and Reynolds et al. (1974) to be about 25 cal/mol. 1 Å^2 would be about a 2% area dilation (assuming the area per lipid molecule is on the order of 50 Å^2). The free energy change per mole of lipid bilayer required to expand the area per molecule by 1 Å^2 would be only on the order of 1 cal/mol, considerably less than pure hydrocarbon-water interaction.

It is also interesting to consider the free energy per mole required to expand the structural matrix to the immediate lysis condition. However, at this point, we must assume that the structural element can be identified. As previously mentioned, the existing evidence implies that the spectrin component which makes up about 35% of the total membrane protein forms the supporting structure or "scaffolding" for the membrane.[4] For a molecular weight of 2×10^5, the concentration of spectrin per square centimeter of membrane is about 5×10^{-13} mol/cm^2. Using the structural network compressibility modulus that was previously calculated,

$$\Delta W_N = (K_N \cdot \Delta\alpha^2)/2,$$

$$\Delta W_N \simeq (193)(0.03)^2(2.4 \times 10^{-8})/(1 \times 10^{-12}),$$

$$\Delta W_N \sim 4 \text{ kcal/mol}.$$

Because of the reported calcium, ATP effects on red cell membrane (e.g. in metabolic depletion) (Weed et al., 1969), it is suggested that measurements of membrane area compressibility in these experiments may provide insight into the physical chemistry of the structural matrix cohesion and its dependence on membrane energy sources.

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[4] The weight percent of spectrin was obtained as an estimate by private communication with Dr. J. Reynolds at Duke University.

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